Prothrombin and fibrinogen carbonylation: How that can affect the blood clotting

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Objectives: The aim of the work was the development of a simple method for measuring the plasma prothrombin carbonylation and the study the impact of prothrombin and fibrinogen oxidation on the rate of plasma clotting.

Methods: A new method was based on the ability of prothrombin to be adsorbed by the barium sulfate. It consists of four steps: prothrombin mixing with the water suspension of $BaSO_4$; reaction of 2,4-dinitrophenylhydrazine with the $BaSO_4$-bound prothrombin; desorption of prothrombin-2,4-dinitrophenylhydrazone complex from $BaSO_4$ in an alkaline medium; neutralization and reading of the optical absorbance of the complex (λ = 370 nm). The prothrombin/fibrinogen carbonylation and plasma clotting rate *in vitro* in the presence of reactive oxygen species (ROS)-generating agents (0.05–0.8 mM Fe^{2+}/H_2O_2) were monitored.

Results: The plasma volume required for measurement of carbonylated prothrombin was 0.4 ml. High level of linearity and reproducibility was observed ($r = 0.9995$, $P = 0.0005$ – for the protein; $r = 0.9971$, $P = 0.0029$ – for carbonyls). In the intact rats, the concentration of blood plasma prothrombin was 0.355 ± 0.009 mg/ml, and that of carbonyls was 4.94 ± 0.09 nmol/mg.

Discussion: Prothrombin and plasma clotting rate was not affected by low concentrations of ROS (0.05–0.2 mM Fe^{2+}/H_2O_2). The fibrinogen was susceptible to ROS-related effect over all the used range of concentration (0.05–0.8 mM Fe^{2+}/H_2O_2). Carbonylation of fibrinogen did not affect the plasma clotting activity at low ROS concentration (0.05–0.2 mM Fe^{2+}/H_2O_2), however it retarded the clotting at higher ROS (0.2–0.8 mM Fe^{2+}/H_2O_2).

Keywords: Protein carbonyls, Oxidation, Prothrombin, Fibrinogen, Blood clotting

Introduction

Oxidative modification of proteins mainly may lead to the loss of protein functional activities. Protein carbonyl content is actually the most general indicator and by far the most commonly used marker of protein oxidation.[1–5] The accumulation of protein carbonyls has been observed in several human diseases including Alzheimer's disease, diabetes, inflammatory bowel disease, arthritis etc.[2,3]

Nowadays the protein carbonyls measurement is a routinely used approach to quantify the level of protein oxidative damage and to assess the imbalance of the pro-/antioxidant system. The most popular method of protein carbonyls assay had been suggested by Levine *et al*.[6] This method is based on the reaction of protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH), with the consequent formation of the protein-bound 2,4-dinitrophenylhydrazones (DNP) hydrazones. These can be measured spectrophotometrically or, with greater sensitivity, immunochemically using anti-dinitrophenyl antibodies.[7] In order to increase the sensitivity of such measurements, several immunoassay approaches (DNPH-based Enzyme-Linked Immuno-sorbent Assay, dot blot, and Western blot) have been developed, and some commercial kits are available today.[8,9] HPLC-separation with electrochemical detection of hydrazones can also be used.[10]

The analysis of oxidative modifications in specific proteins is the most informative approach in investigation of the role of oxidative stress in the development of certain pathology. The joint use of

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anti-dinitrophenyl antibody and antibody to specific protein would probably allow to solve such a problem. However, this approach is complicated and would impact negatively the price of the test. So, the finding of simple, fast and inexpensive method for the evaluation of carbonylation of individual protein seems urgent. The development of such methods might be based on the physicochemical properties of proteins, which are underlie the protein isolation and purification techniques. Undoubtedly, this approach is not suitable for the low-abundance proteins. However, if the content of a specific protein in the biological sample is substantial, its carbonylation can be determined by combining the isolation technique with spectrophotometric measurement of DNP.

In the present work, a new method for the determination of the plasma prothrombin carbonylation is described. This method is based on prothrombin adsorption onto barium sulfate, which was followed by DNP formation. The carbonylation state of the prothrombin that was released by $BaSO_4$ was evaluated spectrophotometrically.

Prothrombin (also called coagulation factor II) is one of the key proteins in the blood coagulation system. After enzymatic cleavage, prothrombin is converted to the active form – thrombin (factor IIa), catalyzing the conversion of fibrinogen to fibrin, thus ensuring clot formation.[11] Slow coagulation rate of oxidatively damaged fibrinogen was reported previously.[12–14] However, we did not find any data concerning the level of prothrombin carbonylation and its impact on the hemostasis rate.

The aim of this study was to develop a simple method for prothrombin carbonylation measurement in blood plasma, and to analyze the impact of the oxidatively damaged prothrombin and fibrinogen on the plasma clotting rate.

Methods

Animals and blood collection

Male outbreed albino rats ranging in weight from 180 to 200 g were used. Blood was collected by heart puncture with 3.8% sodium citrate-containing syringes (9:1, v:v). Samples were, then, centrifuged ($500 \times g$, 10 minutes). In order to monitor the impact of prothrombin and fibrinogen carbonylation on the plasma clotting rate, an aliquot of plasma was incubated for 120 minutes with 0.05–0.8 mM Fe^{2+}/H_2O_2 (Fenton system).[15] This sample was divided to three parts and assayed for prothrombin/fibrinogen carbonylation and plasma clotting.

Prothrombin carbonylation assay

$BaSO_4$ adsorption was used for getting the crude prothrombin preparation.[16] Equal volumes of citrated plasma aliquot and 3% water suspension of $BaSO_4$ (Sigma, Taufkirchen, Germany) (0.05; 0.1; 0.2; 0.4; 0.6; 0.8; 1.0 ml) were mixed. The mixture was incubated for 60 minutes at room temperature (25°C) under shaking conditions, and then centrifuged at $1500 \times g$ for 10 minutes. The supernatant was discarded. The sediment that was formed by prothrombin-$BaSO_4$ complex was washed three times with 5.0 ml of 1.0 mM sodium citrate. The crude prothrombin was not eluted from the $BaSO_4$ pellet. The reaction of DNPH with proteins in the pellet was applied as described earlier.[6] Further, sodium hydroxide (0.5 ml; 0.5 M) was added to the pellet (boiling water bath, 10 minutes) for desorption of prothrombin in accordance with the reaction:

$$\text{Prothrombin} - BaSO_4 + 2NaOH \rightarrow \text{Prothrombin} + Ba(OH)_2 + Na_2SO_4$$

Finally, 2.5 ml of 8 M urea solution in 0.2 M HCl was added to dissolve the protein and to lower the pH (neutralization step). After centrifugation, the absorption spectrum of the supernatant was registered in the electromagnetic range between 230 and 600 nm, using a Hitachi 150-20 UV–VIS spectrophotometer. The amount of generated DNP was monitored at 370 nm (extinction coefficient of 22 000 M^{-1} cm^{-1}).[6] Protein concentration was determined by the biuret method, using a calibration curve that was obtained from bovine serum albumin standards. The purity of prothrombin in the supernatant ($BaSO_4$ eluate) was analyzed by PAAG electrophoresis (7.5%, w/v) in non-denaturing conditions, using Bio-Rad Mini-PROTEAN Tetra System.[17] The molecular mass of the isolated prothrombin was determined in 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[18]

Fibrinogen carbonylation assay

Fibrinogen was precipitated from 0.1 ml of citrated plasma by the addition of 12.7% (w/v) aqueous sodium sulfate (Na_2SO_4) solution, up to final concentration of 10.67%.[19] The solution was, then, incubated at 37°C for 2 hours and then centrifuged at $2000 \times g$ for 10 minutes. The pellet was washed three times with 5 ml of 10.67% Na_2SO_4 and mixed with 1 ml of 20 mM 2.4-DNPH.[6] The amount of DNPs formed and protein concentration were measured as described above. The purity of fibrinogen was analyzed by non-denaturing PAGE (7.5%, w/v).[17]

Plasma clotting assay

Plasma recalcification time (PRCT) was assayed by mixing an equal volume of $CaCl_2$ 20 mM to the citrated plasma that was preincubated in a glass tube for 5 minutes at 37°C. The mixture was then incubated at 37°C, and the clot formation time was established by visual inspection.

Statistical analysis

The statistical analysis was performed using GraphPad InStat software (GraphPad Software, Inc., San Diego, CA, USA, www.graphpad.com). In particular, linear regression analyses were performed to determine the minimum amount of plasma that ensured reliable results of the concentration and the carbonylation of prothrombin in plasma. Non-parametric correlation analysis (Spearman r) was applied to characterize the relationship between prothrombin/fibrinogen carbonylation and plasma clotting rate. Five independent experiments ($n = 5$) were carried out and the results were expressed as means ± standard errors. $P < 0.05$ was considered as statistically significant.

Results

Prothrombin carbonylation assay

A single band of 80–90 kDa MW was shown by the SDS-PAGE performed on the $BaSO_4$ eluate (Fig. 1A). The purity of prothrombin was also demonstrated through non-denaturing PAGE. It was demonstrated that isolated protein was included in the α2-globulins fraction (Fig. 1B). The spectral analysis revealed several absorbance picks. The absorbance maximum of prothrombin (278 nm) was detected both in the control and DNPH-treated samples. This peak is typical for aromatic aminoacids (tyrosine, tryptophane, and phenylalanine) of protein.[20] In differential spectrum two peaks at 262 and 367 nm were observed (Fig. 2). These are typical for DNP produced in the reaction of DNPH with carbonyls of prothrombin and were assigned to π–π* and n–π* transitions of the aromatic compound (DNP).[21]

The measurement of the protein content and carbonylation (Fig. 3) showed high variability and non-reproducibility of the data in the samples from 0.05 to 0.20 ml of plasma. These volumes of plasma brought to falsely overestimated data. On the other hand, high linearity of the absorbance and consistency of the final results (protein or carbonyls final concentration) was shown in the plasma samples 0.40–1.00 ml. The analysis of these samples provided the following data: 0.355 ± 0.009 mg protein/ml (values recalculated taking into account the prothrombin content in plasma) and 4.94 ± 0.09 nmol carbonyls/mg. The linear regression analysis of the 540 nm (biuret) and 370 nm (carbonyls) revealed significant correlation with initial plasma volumes (0.40–1.00 ml) ($r = 0.9995$, $P = 0.0005$ and $r = 0.9971$, $P = 0.0029$ for biuret and carbonyls, respectively).

In order to qualify the advantage of DNPH reaction with $BaSO_4$-bound prothrombin, a reaction with prothrombin that was eluted from $BaSO_4$ (with 100 mM sodium citrate) was carried out. Results are represented as linear regression curves for the samples 0.40–1.00 ml of plasma (Fig. 4). Linear regression data obtained for $BaSO_4$-bound prothrombin ($r = 0.9971$; $P = 0.0029$) were better than those obtained on the prothrombin that was eluted from $BaSO_4$ before the reaction ($r = 0.9129$; $P = 0.081$).

Effects of ROS on prothrombin and fibrinogen carbonylation in vitro

Non-denaturing PAGE revealed that the purity of the separated fibrinogen was sufficient. A single band near the γ-globulins fraction was observed (Fig. 1B). The

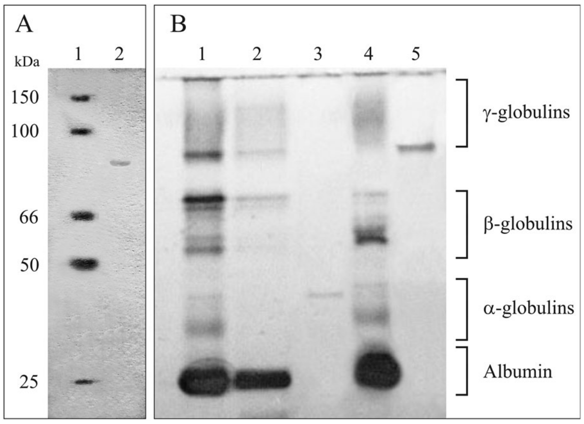

Figure 1 Polyacrylamide gel electrophoresis of prothrombin and fibrinogen. A – SDS-PAGE electropherogram of prothrombin ($BaSO_4$ eluate). Line 1 – molecular mass markers; Line 2 – prothrombin. B – non-denaturing PAGE of prothrombin and fibrinogen. Line 1 – rat's blood plasma; Line 2 – supernatant after proteins adsorbtion on the $BaSO_4$ (1.5% final); 3 – prothrombin ($BaSO_4$ eluate); Line 4 – supernatant after proteins sedimentation in Na_2SO_4 (10.67% final); Line 5 – fibrinogen (Na_2SO_4-precipitate dissolved in PBS 10 mM, pH 7.4).

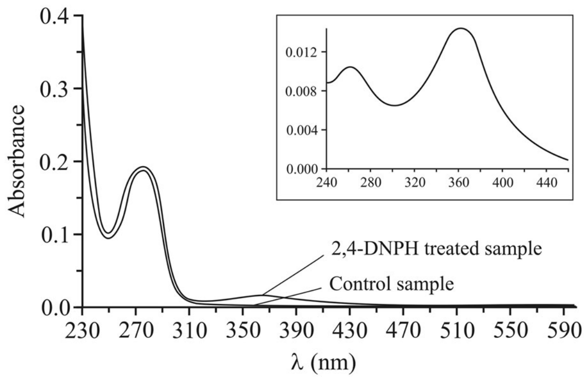

Figure 2 Optical absorption spectra of 2,4-dinitrophenylhidrazone (DNP) derivative of prothrombin. Prothrombin was isolated from 1.0 ml plasma and treated with 2,4-DNPH as described in the methods. Urea solution (8 M) was used as a blank. In control samples, all the steps of carbonylation assay procedure were retained, with the exception of the addition of 2,4-DNPH. The inset shows the differential spectrum (2,4-DNPH-treated control).

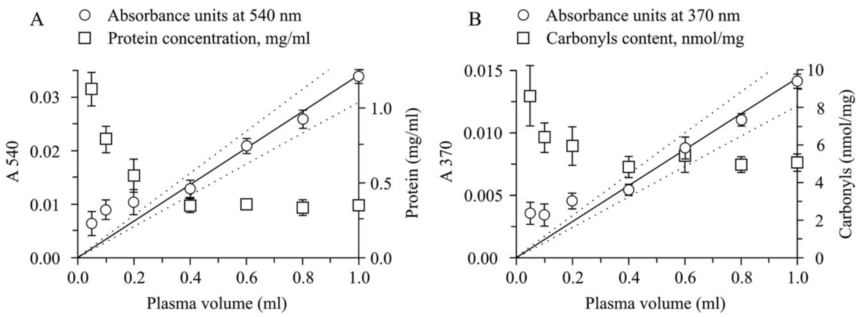

Figure 3 **Minimal plasma volume applicable for prothrombin carbonylation assay. Different volumes of plasma (0.05; 0.1; 0.2; 0.4; 0.6; 0.8; 1.0 ml) were used to establish a linearity range. A – linear regression fit for biuret: absorbance ($\lambda = 540$ nm) vs. initial plasma volume ($r = 0.9995$; $P = 0.0005$) and prothrombin content in plasma. The prothrombin content was calculated using appropriate calibration curve for biuret. B – linear regression fit for carbonyls (DNP): absorbance ($\lambda = 370$ nm) vs. initial plasma volume ($r = 0.9971$; $P = 0.0029$) and carbonyls content in prothrombin. Prothrombin carbonylation was quantified using the extinction coefficient of 22 000 M^{-1} cm^{-1}. Protein concentration was calculated using the calibration curve with the bovine serum albumin. $n = 5$.**

dynamics of prothrombin/fibrinogen carbonylation and plasma clotting velocity (PRCT) was traced *in vitro* after treatment with ROS generated agents (0.05–0.80 mM of Fe^{2+}/H_2O_2). We showed that low concentrations of ROS (0.05–0.20 mM Fe^{2+}/H_2O_2) did not affect significantly the level of prothrombin carbonylation and plasma clotting activity (Fig. 5A), whereas the prothrombin carbonylation was sharply increased in the interval of 0.20–0.40 mM of Fe^{2+}/H_2O_2, reaching a plateau. On the other hand, plasma clotting rate gradually increased in the range of 0.20–0.80 mM Fe^{2+}/H_2O_2. In the same time, the fibrinogen was susceptible both to low and high ROS concentrations. A gradual increase of fibrinogen carbonylation was observed concurrently to the increase of Fe^{2+}/H_2O_2 concentration in the whole range used (0.05–0.8 mM). Non-parametric correlation (Spearman *r*) analysis between protein carbonylation and plasma coagulation velocity revealed higher correlation values between PRCT and prothrombin oxidation ($r = 0.9409$; $P < 0.0001$) obtained using lower ROS concentrations – 0.05–0.20 mM of Fe^{2+}/H_2O_2 (Fig. 5B), and between PRCT and fibrinogen oxidation ($r = 0.9487$; $P = 0.0004$) obtained using higher concentrations of ROS-generating agents – 0.20–0.80 mM (Fig. 5E).

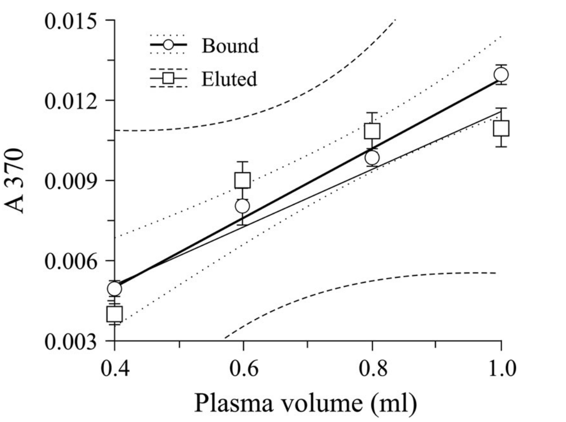

Figure 4 **Data on the reaction of DNPH with $BaSO_4$-bound and eluted prothrombin. Linear regression fit for carbonyls (DNP): absorbance ($\lambda = 370$ nm) vs. initial plasma volume. Bound – $BaSO_4$-bound prothrombin was used for carbonylation assay ($r = 0.9971$; $P = 0.0029$); Eluted – prothrombin eluted from $BaSO_4$ was used for carbonylation assay ($r = 0.9129$; $P = 0.081$). $n = 5$.**

Discussion

Prothrombin isolation and purification technique includes barium salts adsorption and elution, ion-exchange chromatography and gel filtration steps with final yield of prothrombin about 50%.[16,22] In order to simplify the purification process and minimize the loss of the protein, a new method was restricted to $BaSO_4$ adsorption followed the removal of unbound proteins. As shown above, it was enough for the subsequent detection of prothrombin carbonyls. Other vitamin K-dependent proteins (VKDPs) adsorption on $BaSO_4$ was anticipated.[23] Nevertheless, prothrombin concentration in plasma is much higher than those of VKDPs.[24] This aspect strengthens the idea that other VKDPs would not hinder the assay, especially if prothrombin is purified from small plasma volumes (less than 1.0 ml). Indeed, single band of 80–90 kDa was observed in SDS-PAGE. This is in good accordance with rat prothrombin MW (86 kDa) reported by Li and Olson.[16] Prothrombin identity and purity were also proved by native PAGE. A single band was observed in the a2-globulins fraction, which is typical for prothrombin.[25] Concentrations of other VKDPs were probably below the sensitivity of PAGE method, thus, their contribution to the final result of the proposed method was negligible.

Several peaks characteristic for protein (278 nm) and DNP (262 and 367 nm) were observed in the absorbance spectra of prothrombin-DNP adduct, which indicates the reliability of the suggested

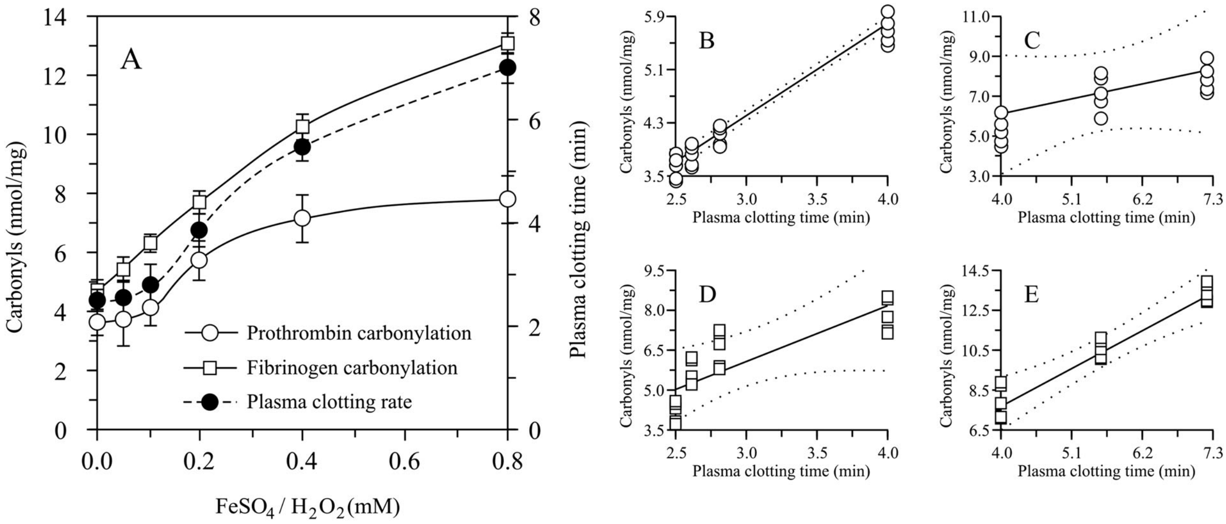

Figure 5 ROS effect on prothrombin/fibrinogen carbonylation and plasma clotting rate. ROS were generated by adding different concentrations of Fe^{2+}/H_2O_2 (0.05–0.8 mM final). A – dynamics of protein carbonylation and plasma clotting rate in the presence of increasing concentrations of oxidizing agents; B – correlation between prothrombin carbonylation and plasma clotting rate in poor ROS-generating conditions (0.05–0.20 mM of Fe^{2+}/H_2O_2): $r = 0.9409$; $P < 0.0001$; C – correlation between prothrombin carbonylation and plasma clotting rate in ROS-enriched conditions (0.20–0.80 mM of Fe^{2+}/H_2O_2): $r = 0.8433$; $P = 0.0061$; D – Correlation between fibrinogen carbonylation and plasma clotting rate in poor ROS-generating conditions: $r = 0.8853$; $P = 0.0003$; E – Correlation between fibrinogen carbonylation and plasma clotting rate in ROS-enriched conditions: $r = 0.9487$; $P = 0.0004$. $n = 5$

approach for the carbonylation assay. The good linearity of the assay was shown in the range of the initial plasma volume of 0.4–1.0 ml.

The protein loss due to washing is probably the main hindrance for the measurement of carbonylation of the protein, when it is present in low concentration. This obstacle was fixed by adding DNPH to the pellet of prothrombin adsorbed on $BaSO_4$. This modification results in increasing the reproducibility and linearity of the assay, as compared to the reaction carried out with dissolved prothrombin eluted from $BaSO_4$ prior to the reaction.

The threshold of prothrombin sensitivity to low concentrations of ROS has been detected (0.20 mM). This was the same threshold for the plasma clotting rate. On the other hand, fibrinogen was shown to be susceptible to low ROS concentrations (0.05 mM), nevertheless this phenomenon was not reflected on the plasma clotting rate. This probably means that with low ROS concentrations, plasma clotting is not affected by oxidized fibrinogen because the prothrombin remains undamaged. On the other hand, with higher ROS concentrations, plasma clotting rate was shown to be affected, which was due to the oxidative damage of the fibrinogen, but not of the prothrombin. Thus, it might be assumed, that prothrombin remains unaffected in mild oxidative stress conditions, and this could be reflected to the prevention of major flaws of the hemostasis rate.

Conclusions

A new method to assay plasma prothrombin carbonylation was developed. This involved prothrombin adsorption on $BaSO_4$, and removal of unadsorbed proteins, as well as the reaction of DNPH with the $BaSO_4$-bound proteins. This method is characterized by simplicity, rapidness and high linearity. Prothrombin was shown susceptible to ROS (no less than 0.20 mM Fe^{2+}/H_2O_2) and this impacted negatively on plasma clotting activity. On the other hand, fibrinogen carbonylation did not affect the plasma clotting activity with low ROS concentration, unless it is heavily damaged.

Disclaimer statements

Contributors None.

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Conflict of interest No conflict of interests.

Ethics approval None

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